Applicants described in a variety of ways the types of inhibitors of calpastatin that could be used in the claimed methods and vectors. An example from the specification is reproduced below:

Page 7, lines 7-20

> In a specific embodiment of the present invention, all or a part of calpastatin, or a nucleic acid encoding all or a part of calpastatin is used as inhibitor. Still more particularly, a peptide comprising all or part of the sequence SEQ ID No. 1 or of a derivative thereof is used.
> As regards more particularly the derivatives, there may be mentioned, by way of example, the compound of sequence SEQ ID No. 2, which corresponds to a fragment of calpastatin. There is advantageously used any derivative composed of the sequence SEQ ID No. 1 or 2 which is capable of specifically or preferentially inhibiting the degradation of the wild-type p53 protein by calpain.

Applicants submit that this clearly and unequivocally describes the "parts" or fragments of calpastatin recited in the claims. One skilled in the art clearly knows what a part of the specifically listed SEQ ID NO: 1 is and how to make it and use it according to the rest of the disclosure. Furthermore, the inclusion of a particular "part" of SEQ ID NO: 1 that is specifically listed as SEQ ID NO: 2 represents unequivocal evidence that a description that one of skill in the art understands is included in the specification.

In addition, original claim 7 reads:

> 7. Use [of a compound capable of modulating the activity of calpain] according to one of the preceding claims, characterized in that the compound is a nucleic acid encoding all or part of calpastatin.

This indicates that applicants intended to claim the recited parts of calpastatin as an embodiment of their invention. Thus, one of skill in the art would not question that applicants both described the "parts" of calpastatin in the specification and claimed them as part of their invention. One of skill in the art also would not question that applicants had possession of the sequences for making and using them.

Beyond these clear disclosures of the structure of a calpastatin and parts thereof, applicants also clearly described how to make and use parts of calpastatin or other inhibitors of calpain in accordance with the invention. Two examples using what would be a routine task, after applicants' disclosure in this specification, of the detection or monitoring of p53 degradation are reproduced below.

Page 8, lines 10-18

> Preferably, the modulators used within the framework of the invention are proteins or polypeptides, or nucleic acid sequences encoding these polypeptides or proteins. Still more preferably, the modulatory compounds are proteins or polypeptides which are specific inhibitors of the activity of calpain on the wild-type p53 protein or forms of calpains, modified or otherwise, for specifically degrading the mutated p53 proteins.

Page 22, lines 15-24

> 2.2 Use of calpain inhibitors to modulate the levels of p53 proteins: the chelation of calcium by EGTA, as well as the use of a whole range of protease inhibitors (leupeptin, aprotinin, soybean trypsin inhibitor and PMSF) and especially the peptide calpastatin show that the degradation of these proteins is dependent on the calpains of the cytoplasmic extract, and that various compounds capable of modulating the activity of the calpains may be used to regulate the p53 protein levels.

From the descriptions above and the earlier descriptions from the specification, at least, one skilled in the art has a clear blue print on what inhibitors of calpain are, how they can be selected and made, how they can be tested for their effect on p53 proteins, and how they can be used to inhibit calpain and inhibit p53 degradation.

The PTO refers to Pfaff v. Wells, 525 U.S. 55, 48 USPQ2d 1641 (1998). That decision points out that one need not actually bring the invention to the highest degree of perfection in order to show that it is ready for patenting, and, indeed, one need not even actually reduce it to practice. ["But just because reduction to practice is sufficient evidence of completion, it does not follow that proof of reduction to practice is necessary in every case. Indeed, both the facts of the *Telephone Cases* and the facts of this case demonstrate that one can prove that an invention is

complete and ready for patenting before it has actually been reduced to practice." Pfaff v. Wells, 525 U.S. 55, 66 (1998).] In fact, the Supreme Court found that the invention was "ready for patenting" in the Pfaff v. Wells case before the inventor had actually made it.

Similarly, applicants need not show with detailed data and examples how every possible fragment or "part" of calpastatin has been reduced to practice before they are entitled to a patent. Applicants have already shown, and the PTO acknowledges, that calpastatin works as disclosed in the specification. One of skill in the art, knowing that polypeptide-based protein inhibitors need not contain the entire or complete amino acid sequence of the polypeptide to retain the inhibition function, would immediately recognize that fragments or parts of calpastatin, as clearly described in the specification, work in the same way. Thus, applicants have provided enough information for one skilled in the art to select, make, and use a part or fragment of calpastatin to inhibit p53 degradation.

As discussed in the Reply filed September 28, 2001, specifically incorporated herein by reference, applicants have met the written description requirement set forth in both the Patent Office Guidelines (66 FR 1099 (Jan. 5, 2001) ("Guidelines for Examination of Patent Applications Under the 35 U.S.C. § 112, ¶ 1, 'Written Description' Requirement") and the relevant cases. Also, applicants have explained why certain fragments may be preferentially selected "parts" of calpastatin to make and use. Finally, a number of documents, including U.S. patent document 5,629,165 (Nixon *et* al., of record) discuss calpastatins, calpastatin proteins and fragments, and a variety of other calpain inhibitors those of skill in the art have made and used as described in applicants' specification (*see also* Atencio, *et al.*, Cell Growth & Differentiation 11: 247-253, copy enclosed).

Applicants request reconsideration and withdrawal of this rejection.

2. Enablement

Claims 18-29 stand rejected under 35 U.S.C. § 112, first paragraph, as the specification allegedly fails to contain a description of subject matter that enables one skilled in the art to make and/or use the invention claimed. Applicants respectfully disagree.

Applicants have shown previously, with arguments and supporting evidence (*see* Reply dated September 28, 2001, specifically incorporated herein by reference) how calpastatin, fragments of calpastatin, and inhibitors of the activity of calpain can be selected and used to regulate p53 levels and inhibit p53 degradation. Furthermore, at least the Atencio *et al.* reference, noted above, additionally evidences that the inhibition of calpain operates as applicants describe in the specification. It would appear that applicants have correctly described methods and vectors that can be used in methods to inhibit p53 degradation within a cell.

Applicants respectfully maintain that the standard being applied here is improper and that the PTO routinely accepts the assertions of enablement for inventions that involve gene transfer or the activity of compounds, such as calpain inhibitors, within a cell. Applicants respectfully request reconsideration.

The PTO points to the Verma, Orkin, Dachs, and Marshall documents and explains that these documents do not base their arguments on clinical trial data, and, thus, the use of these documents as a basis for the enablement rejection is appropriate. However, Verma, for example, begins by discussing only human clinical data at the first two paragraphs. While other factors in gene therapy are discussed for their potential importance, it's clear that the benchmark that Verma considers relevant is human clinical trial success. Similarly, the Marshall document refers to clinical trials in the subtitle and specifically discusses clinical trials and lists certain clinical trials at page 1055. The documents may discuss various technical factors that may be important to certain gene therapy applications. However, the documents also clearly indicate that success in a human clinical trial is the focus of the authors and the story they want to tell.

As shown in the Reply filed September 28, 2001, when the appropriate standard is applied, the PTO has not presented a *prima facie* case of lack of enablement.

Furthermore, applicants submit that numerous publications have demonstrated the scientific merit in applicants' approach to p53 modulation and the inhibition of p53 degradation. Some of the articles submitted indicate as much. In view of this success and the fact that degradation of p53 and modulation of p53 levels is an accepted mechanism in cell biology and the control of cell growth, applicants submit they have met the burden of demonstrating an enabling disclosure. And, as shown above and in the previous Reply, one skilled in the art could

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use routine methods to test for and determine that p53 levels are modified by a particular method or vector selected or used. Thus, no undue experimentation is required to practice the claimed invention.

Applicants respectfully assert that, applying the appropriate standards, one of skill in the art would believe that applicants have enabled the claimed invention. This rejection should be withdrawn.

This application is now in condition for allowance. If the Examiner believes that prosecution might be furthered by discussing the application with applicant's representative, in person or by telephone, we would welcome the opportunity to do so.

Applicants believe that no extension of time fees, requests for extension of time, petitions, or additional claim fees are necessary to enter and consider this paper or keep this application pending. If, however, any extensions of time are required or any fees are due in order to enter or consider this paper or enter or consider any paper accompanying this paper, including fees for net addition of claims, applicants hereby request any extensions or petitions necessary and the Commissioner is hereby authorized to charge our Deposit Account # 50-1129 for any fees. If there is any variance between the fees submitted and any fee required, including the extension of time fee and fee for net addition of claims, the Commissioner is hereby authorized to charge or credit Deposit Account No. 50-1129.

Respectfully submitted,
Wiley Rein & Fielding LLP

Date: March 19, 2002

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WRF 1106270

Calpain Inhibitor 1 Activates p53-dependent Apoptosis in Tumor Cell Lines

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Abstract

Reports suggest a role of calpains in degradation of wild-type p53, which may regulate p53 induction of apoptosis. A calpain inhibitor, n-acetyl-leu-leu-norleucinal (calpain inhibitor 1), was assessed for ability to enhance p53-dependent apoptosis in human tumor cell lines with endogenous wild-type p53 and in altered p53 cell lines with the replacement of wild-type p53 by a recombinant adenovirus (rAd-p53). Calpain inhibitor 1 treatment resulted in increased levels of activated p53, increased p21 protein, and activation of caspases. Cell lines with wild-type, but not mutated or null, p53 status arrested in G_0/G_1 and were sensitive to calpain inhibitor-induced apoptosis. Regardless of endogenous p53 status, calpain inhibitor treatment combined with rAd-p53, but not empty vector virus, enhanced apoptosis in tumor cell lines. These results demonstrate p53-dependent apoptosis induced by a calpain inhibitor and further suggest a role for calpains in the regulation of p53 activity and induction of apoptotic pathways.

Introduction

The tumor suppressor gene product p53 is clearly a central player in many biochemical pathways that are pivotal to human carcinogenesis. The sequence-specific DNA binding properties of this nuclear phosphoprotein regulate the transcription of a continually expanding number of genes, the protein products of which regulate cell cycle progression and apoptosis (1–4). Increasing data support the view that stability of p53 is controlled through mdm2 binding, where binding of p53 to mdm2 has been shown to lead to ubiquitination (5) and subsequent proteolytic degradation of p53 (6).

Although proteasomes have been proposed as the major enzymes involved in p53 degradation (5), calpains, a family of calcium-activated, nonlysosomal neutral proteases, have been proposed to play a role in limited proteolysis of wild-type p53 (7, 8). Calpain inhibitor 1 has been shown to stabilize endogenous p53 in human tumor cell lines independ-

ent of proteasome inhibition (7). There have been reports on apoptosis induced by inhibitors to calpains in prostate tumor cell lines (9). The prostate cell lines used had endogenous mutated p53, however, and the high concentrations of inhibitor used may also have lead to inhibition of proteasomes, which may have resulted in p53-independent apoptosis (10). A recent report on the use of calpain inhibitor 1 to inhibit proteasomes shows the induction of apoptosis independently from p53 (11).

We, therefore, sought to determine whether calpain inhibition in the absence of detectable inhibition of proteasomes could lead to the induction of endogenous and exogenous p53-mediated apoptosis by treating a number of human tumor cell lines with calpain inhibitor 1 alone or in combination with replication-defective rAd[2] expressing wild-type p53 (rAd-p53). Tumor cell lines showed p53-dependent apoptosis with calpain inhibitor 1, alone in wild-type p53 cell lines or in combination with replication-defective rAd-p53. Cells infected with the control virus and treated with calpain inhibitor 1 showed no increase in apoptosis, supporting p53-mediated apoptosis in response to treatment. Calpain inhibitor 1 treatment led to increases in p53 and p21 protein levels, activation of caspases, and did not result in a detectable decrease of NF-κB or AP-1 activity. Sustained NF-κB activity suggests that calpain, rather than proteasome inhibition, was responsible for the effects. Importantly, calpain inhibitor decreased the concentration of rAd-p53 required to induce apoptosis in a p53 altered tumor cell line, suggesting that calpain is involved in suppression of p53-mediated apoptosis in tumor cells.

Results

Effect of Calpain Inhibitor 1 on Cell Cycle. We sought to determine whether tumor cell lines could be arrested in G_0/G_1 in response to calpain inhibitor treatment in a p53-dependent manner. Calpain inhibitor has been reported to arrest human fibroblast cells at the G_1/S phase boundary, where increased p53 levels were detected (12). Among the cell lines analyzed were hepatocellular carcinoma lines (SK-HEP-1 and HLF) and colorectal cell lines (RKO and DLD-1), which differ in their p53 status. At high concentrations (50–100 μM) calpain inhibitor 1 (n-acetyl-leu-leu-norleucinal) has been reported to inactivate proteasomes and lead to a G_2/M arrest (13). However, at lower concentrations (1–20 μM), calpain inhibitor 1 does not show significant effects on proteasomes (7), and at 5 μM calpain inhibitor 1, no G_2/M arrest was detected (data not shown). The inhibition of BrdUrd incor-

[2] The abbreviations used are: rAd, recombinant adenovirus; BrdUrd, bromodeoxyuridine; NF-κB, nuclear factor-κB; AP-1, activator protein-1; ATCC, American Type Culture Collection; DMF, dimethylformamide; CMV, cytomegalovirus.
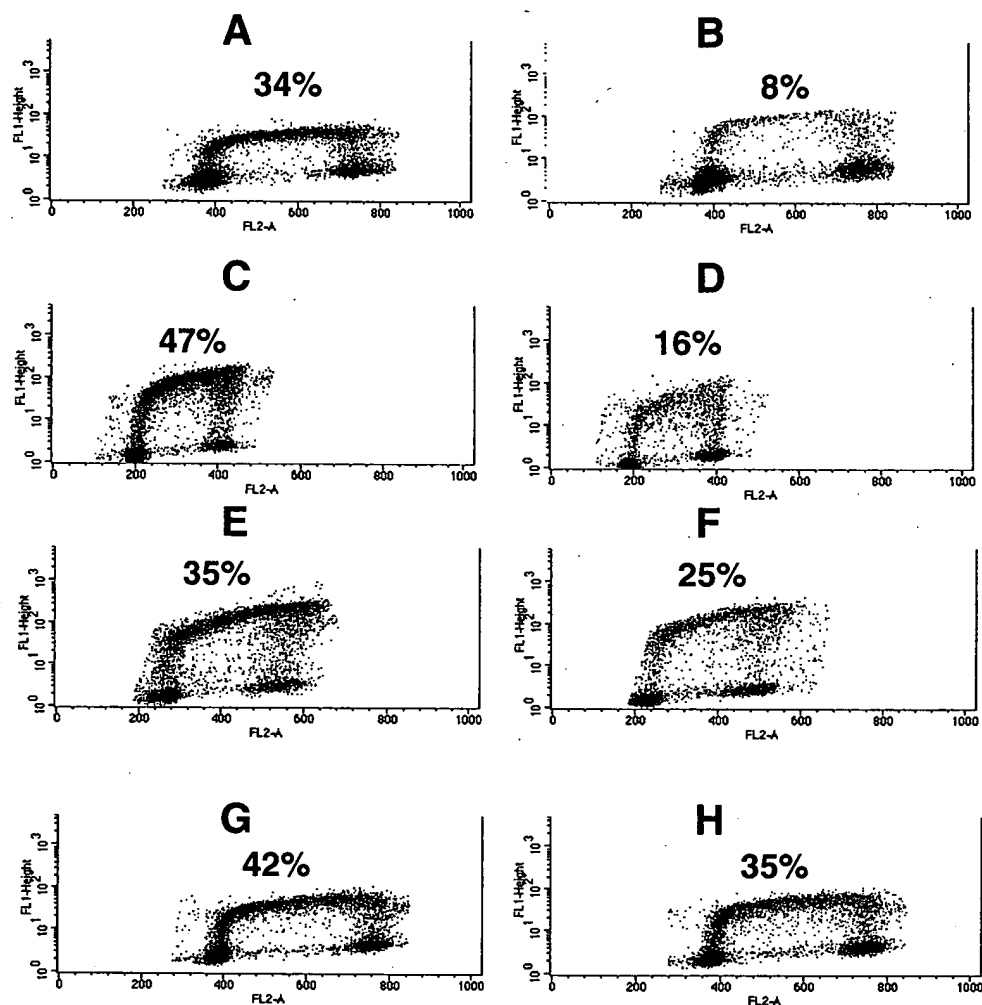
Fig. 1. Cell cycle analysis of cell lines treated with calpain inhibitor 1. Shown is bivariate BrdUrd/DNA flow cytometric analysis of cell lines with endogenous wild-type p53, SK-HEP-1 (*A* and *B*) and RKO (*C* and *D*), and cell lines with mutated p53, DLD-1 (*E* and *F*) and HLF (*G* and *H*) treated with DMF alone (*A, C, E,* and *G*) or 5 μM calpain inhibitor 1 (*B, D, F,* and *H*) for 17 h and pulsed with BrdUrd for 2 h. The percentage of cells that incorporated BrdUrd are indicated in each plot.

poration was measured by flow cytometric analysis to detect a G_0/G_1 block 17 h after calpain inhibitor treatment. In response to calpain inhibitor 1, cell lines with p53 wild-type, but not mutated or null status, were induced to undergo cell cycle arrest (Fig. 1). In response to calpain inhibitor 1, tumor cell lines with endogenous wild-type p53 dramatically decreased the percentage of cells incorporating BrdUrd. For SK-HEP-1, BrdUrd incorporation decreased from 34% (Fig. 1*A*) to 8% (Fig. 1*B*) and from 47% (Fig. 1*C*) to 16% (Fig. 1*D*) for RKO. In contrast, tumor cells lines with mutated p53 had marginal changes in BrdUrd incorporation after treatment. The percentage of cells incorporating BrdUrd changed from 35% (Fig. 1*E*) to 25% (Fig. 1*F*) for DLD-1 and 42% (Fig. 1*G*) to 35% (Fig. 1*H*) for HLF cells treated with calpain inhibitor 1. Thus, calpain inhibitor treatment caused p53-dependent cell cycle arrest.

Effect of Calpain Inhibitor 1 on Induction of Apoptosis. To determine whether inhibition of calpain activity could induce a p53-mediated apoptotic response, tumor cells were treated with increasing doses of calpain inhibitor 1. Annexin V-FITC staining, followed by flow cytometric analysis, was used to quantitate the percentage of cells undergoing apoptosis in response to calpain inhibitor 1. A dose-dependent increase in apoptotic cells was observed only in cell lines with wild-type p53 (Fig. 2). Propidium iodide staining of these cells confirmed subgenomic populations, and visualization of the cultures typically revealed blebbed nuclei and loss of plate adherence, characteristics of apoptosis (data not shown). Other p53 wild-type cell lines treated with calpain inhibitor 1 included the glioblastoma cell line U-87 MG, which showed an increase in the percentage of apoptotic cells from 5% to 72% in response to 10 μM calpain inhibitor 1 treatment (data not shown). In contrast, a lung cell carcinoma line with p53 mutated status, NCI-H596, and a p53 null hepatocellular carcinoma line, Hep3B, showed no increase in the percentage of cells that were Annexin V positive (data not shown) in response to 50 μM calpain inhibitor 1.
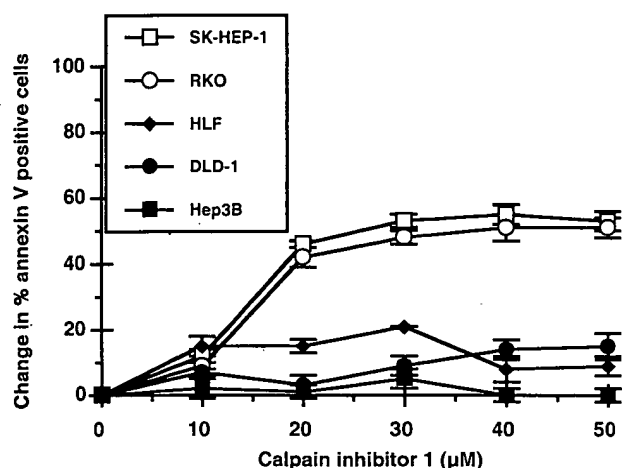
Fig. 2. Effect of calpain inhibitor 1 on induction of apoptosis. Cell lines, SK-HEP-1 (□), RKO (○), HLF (◆), DLD-1 (●), and Hep3B (■) were treated with increasing doses of calpain inhibitor 1 for 24 h. Each data point represents the percentage of Annexin V-FITC-positive cells after the background percentage (DMF alone) of each cell line was subtracted. *Bars,* SD from two independent experiments.

Fig. 3. Effect of rAd-p53 treatment in combination with calpain inhibitor 1. The percentage of cells undergoing apoptosis was measured by Annexin V-FITC staining, followed by flow cytometric analysis. DMF, calpain inhibitor 1 (at 10 μM concentration for DLD-1 and HLF, and at 5 and 20 μM concentration for SK-HEP-1 and RKO respectively), rAd-p53 (1 × 10^9 particle number/ml for DLD-1, HLF, and at 2 × 10^9 particle number/ml for SK-HEP-1), control virus (1 × 10^9 particle number/ml for DLD-1, HLF, and RKO, and at 2 × 10^9 particle number/ml for DLD-1, HLF, and RKO, and at 2 × 10^9 particle number/ml for SK-HEP-1) in combination with calpain inhibitor 1, rAd-p53 in combination with calpain inhibitor 1. □, DMF; ▨, calpain inhibitor 1; ▦, rAd-p53; ▤, control virus + calpain inhibitor 1; ■, rAd-p53 + calpain inhibitor 1. *Bars,* SD from two independent experiments.

The specificity of induction of apoptosis by inhibition of μ-calpains, which have been shown to play a more prominent role in p53 proteolysis (7, 8), was assayed by treatment of cells with the inhibitor more specific to m-calpains, *n*-acety-leu-leu-methioninal. No increase in percentage of apoptotic cells was seen 26 h after treatment of RKO and SK-HEP-1 cells treated with up to 50 μM m-calpain inhibitor (data not shown), suggesting that the apoptotic effect was specific to inhibition of μ-calpain.

Effect of rAd-p53 Infection in Combination with Calpain Inhibitor 1. To further demonstrate that inhibition of calpain activity caused p53-dependent apoptosis, we expressed wild-type p53 in tumor cell lines in combination with calpain inhibitor. An E1 deleted recombinant adenovirus with the CMV promoter expressing p53 was used to express wild-type p53 (rAd-p53) in tumor cells in combination with calpain inhibitor treatment. Independent of p53 status, calpain inhibitor 1 in combination with rAd-p53 infection significantly increased apoptosis at 17 h after treatment, whereas no significant death was seen at these concentrations of inhibitor or virus alone. As shown in Fig. 3, although the concentrations of rAd-p53 and calpain 1 inhibitor needed to induce apoptosis differed among cell lines, all cell lines treated with calpain inhibitor 1 in combination with rAd-p53 showed increases in percentage of apoptotic cells above that seen with either the inhibitor or the virus alone. This shows that cell lines not sensitive to calpain inhibitor-induced apoptosis, p53-altered lines, became sensitive to calpain inhibitor with the reintroduction of wild-type p53. In cell lines DLD-1, RKO, and HLF, the effects were concentration dependent. The majority of SK-HEP-1 cells were apoptotic with 5 μM calpain inhibitor 1, and a similar observation was made with the glioblastoma cell line U-87 MG (data not shown), where both lines were extremely sensitive to the apoptotic effects of rAd-p53 and calpain inhibitor 1.
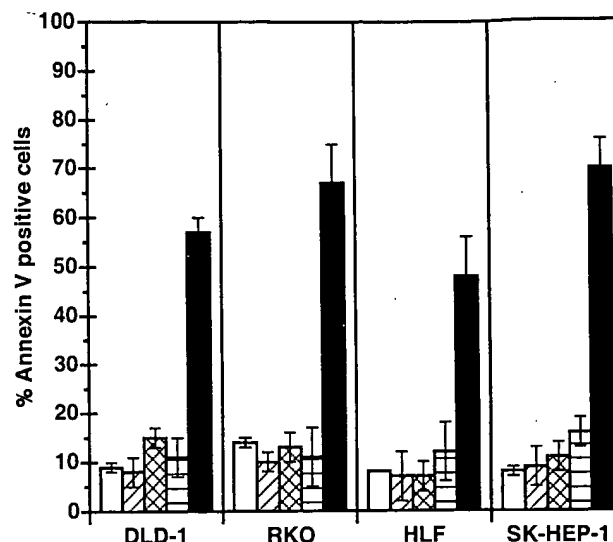
A control virus encoding no transgene in combination with calpain inhibitor 1 showed no significant increase in cell death above that seen with the inhibitor alone. For the cell lines DLD-1, HLF, RKO, and SK-HEP-1, the percentages that were annexin V positive were 9, 8, 14, and 8%, respectively. These results demonstrate a specific p53 transgene effect.

Western Blot Analysis for p53 and p21 Levels. Inhibition of calpain activity is expected to cause increased p53 protein levels (7, 8) and therefore increased p21 protein levels attributable to transcriptional activation of the p21 promoter by p53 (14). Hep3B cells with p53 null status were treated with rAd-p53 at 1 × 10^9 particle number/ml for 1 h and then treated with 10 μM calpain inhibitor 1 for 17 h. As shown in Fig. 4, Western blot analysis revealed that in response to rAd-p53, cells showed increases in p53 and p21 protein levels. When 10 μM calpain inhibitor 1 was added, the levels of p53 and p21 increased ~3-fold. Similar 3–5-fold increases in p53 and p21 protein levels were seen with cell lines SK-HEP-1, DLD-1, and RKO (data not shown), indicating that calpain inhibitor was able to increase the exogenous p53 and that the increased p53 protein levels could lead to enhancement of downstream events, such as increased p21 protein. The ability of calpain inhibitor 1 to increase endogenous p53 and p21 levels was assessed by using the p53 wild-type cell line, SK-HEP-1. In response to 5 and 10 μM calpain inhibitor 1, an increase of 5–10-fold in p53 and p21 protein levels occurred.

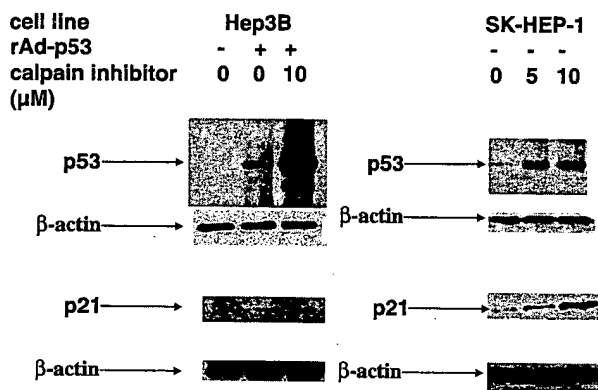| cell line	Hep3B			SK-HEP-1		
rAd-p53	-	+	+	-	-	-
calpain inhibitor (μM)	0	0	10	0	5	10

Fig. 4. Western blot analysis for p53 and p21 in tumor cell lines treated with DMF (−), infected with rAd-p53 (+) alone or in combination with 5 μM (5) or 10 μM (10) calpain inhibitor 1 for 17 h. Western blots were quantitated by NIH Image.
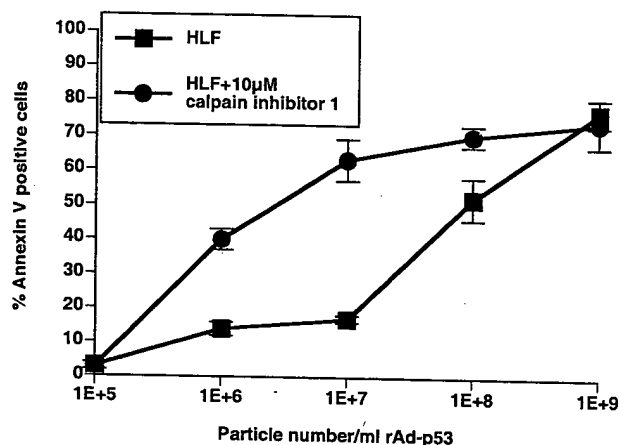

Fig. 5. rAd-p53 dose response curve to the induction of apoptosis. HLF cells were treated with 10 μM calpain inhibitor 1 (●) or DMF (■) and increasing doses of rAd-p53. The percentage of cells undergoing apoptosis was determined by Annexin V-FITC staining, followed by flow cytometric analysis. *Bars*, SD from two independent experiments.

rAd-p53 Dose Response Curve to the Induction of Apoptosis in the Presence or Absence of Calpain Inhibitor 1. To better quantitate the increased efficacy of rAd-p53-induced cell killing in tumor cell lines in response to calpain inhibitor 1 treatment, HLF cells were infected with increasing concentrations of rAd-p53 with or without 10 μM calpain inhibitor 1 and assayed for apoptosis by annexin V-FITC staining 42 h after treatment. The dose response curve was plotted and is shown in Fig. 5. To induce cell death in 50% of the HLF cells, 1×10^8 particle/ml concentration of rAd-p53 was needed without calpain inhibitor 1, whereas only a 3×10^6 particle/ml of rAd-p53 were required with 10 μM calpain inhibitor 1, representing >10-fold decrease in the concentration of rAd-p53 needed to induce ~50% cell death.

Activation of NF-κB and AP-1 in rAd-p53-infected Cells in Response to Calpain Inhibitor 1. To confirm that calpain, rather than proteasome activity, was inhibited with calpain inhibitor 1, we assayed for NF-κB activity by gel shift
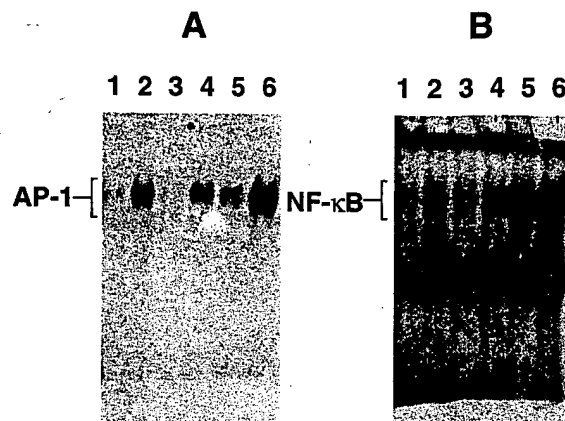

Fig. 6. Gel shift assays for AP-1 (*A*) and NF-κB (*B*) activation in the HLF cell line treated with DMF alone (*Lanes 1A* and *1B*), 10 μM calpain inhibitor 1 (*Lanes 2A* and *2B*), control virus (*Lanes 3A* and *3B*), control virus and calpain inhibitor 1 (*Lanes 4A* and *4B*), rAd-p53 (*Lanes 5A* and *5B*), and rAd-p53 with calpain inhibitor 1 (*Lanes 6A* and *6B*).

assays (15). Because the calpain inhibitor we used has been shown to inactivate proteasomes at high concentration (13), we wanted to eliminate the possibility that the effects we observed were attributable to inactivation of proteasomes. Reports of calpain activity on the proteolysis of NF-κB (16) suggest that inhibiting calpain activity will lead to increased NF-κB activation, whereas inhibiting proteasome function will lead to stabilization of the inhibitor iκBα (17) and hence decrease NF-κB activity (15, 18–20). As shown in Fig. 6*B*, HLF cells treated with 10 μM calpain inhibitor 1 showed no decreased activation of NF-κB (*Lane 2*), as compared with DMF-treated control cells (*Lane 1*). In response to rAd-p53 (*Lane 5*) but not control virus (*Lane 3*), increased NF-κB activation was detected, which was enhanced with calpain inhibitor 1 treatment (*Lane 6*). The activation of NF-κB suggests inhibition of calpain activity, rather than the inhibition of proteasome function, by calpain inhibitor 1 in these studies.

Zhu *et al.* (9) have reported on the role of increased AP-1 activation in the induction of apoptosis in p53 mutated prostate cell lines in response to treatment with calpain inhibitors. To assess a role of increased AP-1 activity in mediating apoptosis in a cell line with mutant p53 status, we treated a p53 mutated cell line, HLF, with 10 μM calpain inhibitor 1 with and without rAd-p53 and assayed for AP-1 activation. As shown in Fig. 6*A*, an increase in AP-1 activity in response to calpain inhibitor 1 was detected by gel shift assay (*Lane 2*), as compared with the DMF control alone (*Lane 1*). A significant increase in AP-1 activity was also detected in response to rAd-p53 alone (*Lane 5*), although not in response to the adenovirus vector expressing no transgene (*Lane 3*). Treatment with the combination of calpain inhibitor and rAd-p53 caused AP-1 activity above the level detected by either alone (*Lane 6*).

Activation of Caspases in Calpain Inhibitor 1-induced Apoptosis. To show that the apoptotic pathway activated by treatment with calpain inhibitor 1 was similar to p53-induced apoptotic pathways, a subset of the family of caspases was assayed in p53 wild-type cells treated with
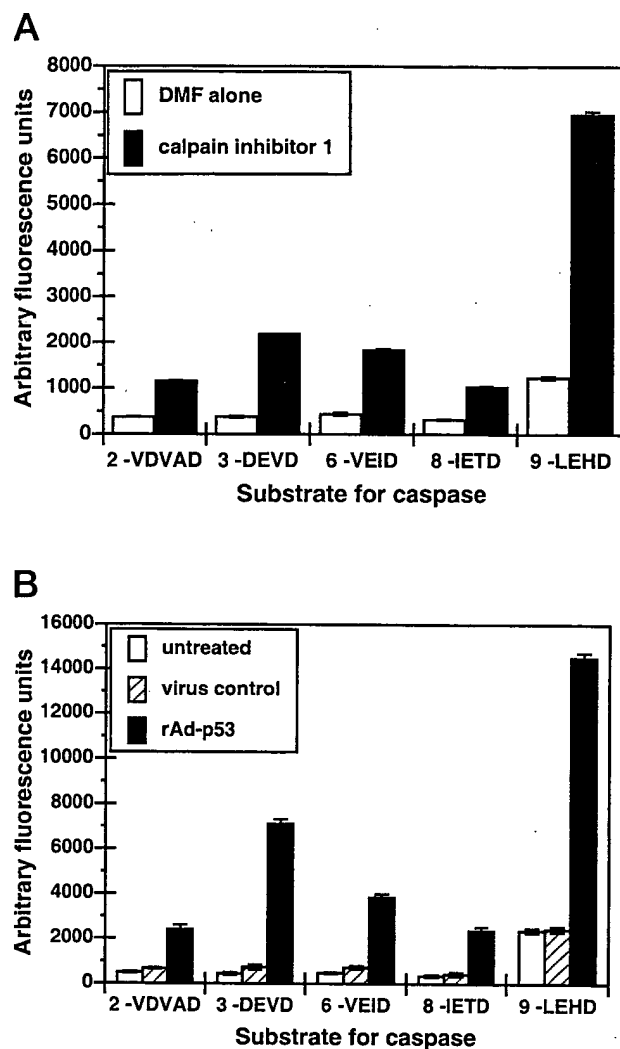
A

B

Fig. 7. Activation of caspases. SK-HEP-1 cells (*A*) were treated with calpain inhibitor 1. HLF cells (*B*) were treated with rAd-p53. Cell lysates were analyzed for caspase activity by incubation with fluorogenic substrates specific for caspase family members 1-YVAD, 2-VDVAD, 3-DEVD, 6-VEID, 8-IETD, or 9-LEHD. *Bars*, SD from two independent experiments.

calpain inhibitor and in p53-altered cells treated with rAd-p53. SK-HEP-1 cells were treated with 5 μM calpain inhibitor 1 for 43 h, at which time apoptotic cells were detected by Annexin V-FITC staining (data not shown). HLF cells were treated with rAd-p53. Cell lysates were analyzed for activity of caspases 1, 2, 3, 6, 8, and 9 by incubating lysates with specific caspase substrates. Either treatment resulted in a 3–7-fold increase in activity of caspases 2, 3, 6, 8, and 9 (Fig. 7). No increase in caspase 1 activity was observed in either treatment (data not shown). The calpain inhibitor, therefore, induced activation of caspases in a similar manner as rAd-p53 reintroduction, supporting the premise that calpain inhibition can lead to p53-mediated apoptosis.

Discussion

Increased stability of p53 protein had been observed as a result of inhibition of calpain activity using calpain inhibitor 1

(7, 8). We have extended this study to show that this inhibitor to calpains can cause p53-dependent cell cycle arrest and apoptosis, with activation of caspases, in a variety of tumor cell lines. All cell lines treated with calpain inhibitor 1 in combination with rAd-p53 showed increases in apoptosis over rAd-p53 or calpain inhibitor 1 treatment alone. Cell lines that were resistant to calpain inhibitor induced apoptosis, p53-altered lines, were sensitive upon the reintroduction of wild-type p53 and demonstrate p53-dependent cell killing with calpain inhibitor. Although high concentrations of calpain inhibitor 1 have been reported to inhibit proteasome function, which also may result in increased p53 protein levels (5), the concentrations of calpain inhibitor 1 we used in this report were not sufficient to inhibit E6-targeted degradation of p53 via the ubiquitin/proteasome pathway (6). In addition, although others have reported decreased NF-κB activity in response to proteasome inhibition because of stabilization of the NF-κB inhibitor iκbα (17–19, 20, 21), we observed no decreased activity in lysate from calpain inhibitor 1-treated cells. These results suggest that inhibition of calpain, rather than proteasomes, activated p53-dependent apoptosis.

We also observed increased p53 protein levels as a result of calpain inhibitor treatment and extended this observation to include increases in p21 protein levels. These results indicate that calpain inhibitor 1 treatment increases levels of exogenous as well as endogenous p53 and were confirmed using a p53 null cell line, Hep3B. Calpain inhibitor 1 treatment in combination with adenovirus-mediated *p53* gene transfer was effective in inducing apoptosis, even when the increase in p53 levels were modest (2–5-fold increases), suggesting that calpain inhibition may make these cells more sensitive to p53-mediated apoptosis, although not necessarily via increases in p53 levels alone.

Addition of calpain inhibitor 1 reduced the concentration of rAd-p53 by 30-fold needed to induce apoptosis, suggesting that in a gene therapy context, the dosage of rAd-p53 delivered to patients could potentially be decreased with the addition of a calpain inhibitor. Additional potential benefits in the use of calpain inhibitors to treat neoplasia may include decreased cell metastasis (22) and increased levels of tumor suppressor factors other than p53, such as tumor suppressor NF2 (23). Transcriptional activation of p53 in cells may in addition lead to activation of p53-responsive genes such as fas (14), providing for sensitization of cells to Fas-mediated apoptosis. Cells may, in addition, become sensitized to other apoptotic stimuli, although we have not addressed this issue in this study. Because calpain inhibitors have been used in a variety of pathological indications to protect cells from death (24–26), we believe the enhancement of cell death observed in this study may be specific for tumor cells.

Interestingly, we observed elevated AP-1 activity in response to rAd-p53 alone and in combination with calpain inhibitor 1. Zhu *et al.* (9) have reported that prostate cell lines with p53 mutated status undergo apoptosis when treated with calpain inhibitor 1, where prolonged AP-1 activity had been observed. We also detected increased AP-1 activity in HLF cells treated with calpain inhibitor alone but did not detect increased cell death at that time point. The combina-

tion of calpain inhibitor and rAd-p53 lead to enhanced AP-1 levels over the effect of either agent alone, suggesting that AP-1 activity may be playing a role in p53-mediated apoptosis. Additional effects on cellular activities have been reported using calpain inhibitor 1, as well as other calpain inhibitors, such as stabilization of factors in cell survival and apoptosis (16, 20) and cell cycle control (27–29).

Although the precise mechanism of inhibition of calpains and p53-mediated apoptosis needs to be delineated, our results further support the hypothesis that calpains may be involved in suppression of p53-mediated apoptosis and demonstrate a potential use of calpain inhibitors as therapeutic agents in the treatment of tumors with wild-type p53 and in combination with rAd-p53 in gene therapy.

Materials and Methods

Cell Lines. SK-HEP-1 (ATCC #HTB-52), DLD-1 (ATCC #CCL-221), Hep3B (Hep3 B2.1–7, ATCC #HB-8064), NCI-H596 (ATCC #HTB-178), and U-87 MG (ATCC #HTB-14) were obtained from the ATCC (Manassas, VA). The HLF line was obtained from the Japanese Cancer Research Resource Bank (Tokyo, Japan), and the RKO line was obtained from M. Brattain (Medical College Hospital, Toledo, OH). All cell lines with the exception of NCI-H596 were grown in DMEM supplemented with sodium pyruvate and 10% fetal bovine serum. NCI-H596 cells were grown in Ham's F-12:DME (1:1) supplemented with glutamine and 10% fetal bovine serum.

Protease Inhibitor Treatment. Calpain inhibitor 1, n-acetyl-leu-leu-norleucinal (Boehringer Mannheim), diluted in DMF was added to cell lines at 5–100 µM final concentration. Control cells were treated the solvent DMF alone. m-calpain inhibitor, n-acetyl-leu-leu-methioninal (Boehringer Mannheim), was added to cell lines at 5–50 µM.

rAd-p53 in Combination with Protease Inhibitor Treatment. Cell lines were infected with 3×10^7–2×10^9 particle number/ml (30) rAd expressing wild-type p53 from a CMV promoter (rAd-p53; Ref. 31) or with a control virus with a CMV promoter but no transgene (32). A pulsed viral infection was performed for 1 h, at which time virus was aspirated and fresh media were added with or without calpain inhibitor at 5, 10, or 20 µM final concentration.

BrdUrd Pulse Labeling of Cells. Cell lines treated with DMF alone or 5 µM concentration of calpain inhibitor 1 for 17 hours were pulse labeled with 10 µM BrdUrd for 2 h. Cells were harvested for bivariate BrdUrd/DNA flow cytometric analysis by fixation in 70% ethanol, followed digestion with 0.08% pepsin for 30 min at 37°C. Cells were centrifuged at 1500 rpm, and 2 N HCl was added. Cells were incubated at 37°C for 20 min, followed by addition of 1 M sodium borate. Cells were washed in IFA/Tween 20 (0.01 M HEPES, 0.005% sodium azide, 0.5% Tween 20, 5% fetal bovine serum, and 0.15 M NaCl), and anti-BrdUrd antibody (Becton-Dickinson), diluted 1:10 in IFA without Tween 20, was added for 30 min. Cells were washed in IFA/Tween 20 and incubated in IFA/Tween 20/RNase for 15 min at 37°C and stained with propidium iodide (50 µg/ml).

Apoptosis. Apoptosis was monitored visually by observing blebbed nuclei characteristic of apoptosis, by propidium iodide staining (Molecular Probes, Inc.), followed by flow cytometric analysis to quantitate subgenomic populations of cells, and by labeling cells with Annexin V-FITC (Boehringer Mannheim), followed by flow cytometric analysis (33).

Western Blotting. At 17 h after treatment, cells were lysed in protein lysis buffer (50 mM Tris, 250 mM NaCl, 50 mM NaF, 5 mM EDTA and 0.1% NP40 with 1 mM phenylmethylsulfonyl fluoride). Ten-µg of protein were added per lane on a 12% polyacrylamide gel and transferred onto nitrocellulose membranes. The membranes were subjected to Western blot analysis using antibodies specific for p53 or p21 (Calbiochem, p53 antibody Ab-6, and p21 antibody Ab-7). Blots were incubated for 1 h in horseradish peroxidase-conjugated secondary antibodies. Blots were developed using the enhanced chemiluminescence detection system (Amersham) and quantitated using NIH Image software.

Gel Shift Assays for NF-κB and AP-1. Double-stranded oligonucleotides containing high-affinity binding sites for NF-κB (Promega E329) or AP-1 (Promega E320) were labeled using [γ-^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase and purified using MicroSpin G-25 columns. Nuclear extracts (1 µg of protein) were incubated in a 10-µl (final volume) reaction mixture containing 10 mM Tris-HCl (pH 7.5), 0.5 mM DTT, 0.5 mM EDTA, 1 mM MgCl$_2$, 4% glycerol, and 50 ng/ml poly deoxyinosinic-deoxycytidylic acid at room temperature for 10 min. Labeled oligonucleotides (~100,000 cpm) were then added, and the reaction mixtures were incubated for another 20 min at room temperature. After 20 min incubation, 5 µl of 60% glycerol were added to each reaction, and the samples were subjected to native polyacrylamide gel electrophoresis. After electrophoresis, gels were dried and exposed to an X-ray film at −70°C.

Caspase Activation. SK-HEP-1 cells were treated with 5 µM calpain inhibitor 1 for 43 h, at which time apoptosis was detected. HLF cells were infected with 1×10^8 particle number/ml rAd-p53 for 1 h; cells were washed with fresh media and incubated for 24 h, at which time apoptotic cells were detected. 1×10^6 cells/assay were lysed in 50 µl of cell lysis buffer (Clontech, Inc.). To the cell lysates, 20 mM substrate (Enzyme Systems) for caspase 1 (Ac-YVAD-AFC), caspase 2 (Ac-VDVAD-AFC), caspase 3 (Ac-DEVD-AFC), caspase 6 (Ac-VEID-AFC), caspase 8 (Ac-IETD-AFC), or caspase 9 (Ac-LEHD-AFC) was incubated was 37°C for 1 h. Arbitrary fluorescence units was determined on a Cytofluor fluorescence multiwell plate reader (PerSeptive Biosystems) on 400-nm excitation filter and 505-nm emission filter.

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